PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PCT24285		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT00/00048	16/02/2000	22/02/1999
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant UNIVERSITA DEGLI STUDI DI ROMA "LA SAPIENZA"		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the International application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/09/2000	19.07.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer BULCAO DE MELO .., T Telephone No. +49 89 2399 8972

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-37 as originally filed

Claims, No.:

1-23 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

Sequence listing part of the description, pages:

20-37, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-23
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-23
Industrial applicability (IA)	Yes:	Claims	1-23
	No:	Claims	

2. Citations and explanations
   see separate sheet

1. Reference is made to the following document:

   D1: Proc. Natl. Acad. Sci. USA, Vol. 94, pages 10233-10238, 1997

2. The present International Preliminary Examination Report has been established with the assumption that the **priority date** 22.02.99 is validly claimed. Therefore, documents EMBL Sequence Database Accession Numbers AJ133528 (10 May 1999) and Q9Y7G6 (1 November 1999) and Nature, Vol. 399, 13 May 1999, pages 166-169, have not been considered to be part of the prior art as defined in the regulations (**Rule 64 (1) and (3) PCT**).

SECTION V

3. Novelty (**Article 33(2) PCT**)

   The subject-matter of the present application does not appear to be disclosed in the prior art as defined in the regulations (**Rule 64 (1)-(3) PCT**).
   Therefore, in view of such prior art the subject-matter of the present application (**claims 1-23**) has to be regarded as being new (**Article 33 (2) PCT**).

4. Inventive Step (**Article 33 (3) PCT**)

   The present application does not satisfy the criterion set forth in **Article 33 (3) PCT** because the subject-matter of **claims 1-23** does not involve an inventive step (**Rule 65 (1) and (2) PCT**).

   The **closest prior art** to evaluate the inventiveness of the present application is document **D1**. D1 discloses the isolation of *Neurospora crassa* mutants defective in "quelling" or transgene-induced gene silencing. The recessive quelling-defective (*qde*) mutants belong to three complementation groups corresponding to three distinct genes, *qde-1*, *qde-2* and *qde-3*, whose products are essential to the silencing machinery. (See Abstract; page 10236, right hand column-page 10237, left hand column, first paragraph; table 2 and Discussion).

Starting from **D1**, the underlying **technical problem** to be solved by the present application can be considered to lie in the cloning of the *qde* genes.

The **solution** provided by the Applicant to solve the above problem is the *qde-1* gene having sequence SEQ ID NO:1.

D1 suggests that the *qde* mutations may be used, and should provide the first tools, to isolate the genes encoding the first components involved in the quelling mechanism. D1 further strengths that the cloning of the *qde* genes will be of extreme importance to resolving the puzzle of post-transcriptional gene silencing.

In view of the above, the person skilled in the art would be taught to clone the *qde* genes necessary for quelling.
Starting from a *qde* mutant strain, such as the *al-1 Neurospora crassa* transgenic strain which shows an albino (white) phenotype as a consequence of post-transcriptional silencing of the endogenous *al-1* gene (see D1, for example page 10235, left hand column), the person skilled in the art does not require any inventive skills in order to clone a *qde* gene. This is regarded as a current procedure which involves techniques well known in the art.

It should be noted that once the *qde-1* mutants are available, the mutagenesis approach used for their generation is of no relevance for the assessment of an inventive step for the isolation and cloning of said mutants. Moreover, although some methods may provide some advantages in terms of facility of cloning, this does not confer an inventive step to the cloned gene.
What is being claimed is the cloned gene and not a method of mutagenesis nor a method of cloning.

Furthermore, although molecular genetics is an inherently unpredictable art, involving delicate procedures and constantly presenting problems and difficulties, in the present application there is no indication showing that the inventors required inventive skills to overcome any problems or difficulties.

Therefore, the subject-matter of the present application does not involve an inventive step.

5. Industrial Applicability (**Article 33(4) PCT**)

The subject-matter of the present application (**claims 1-23**) is susceptible of industrial applicability as defined in **Article 33 (4) PCT**.

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
PCT24285		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IT 00/ 00048	16/02/2000	22/02/1999
Applicant		
UNIVERSITA DEGLI STUDI DI ROMA "LA SAPIENZA"		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/31 C12N15/63 C12N15/67 C12N15/70 C12N15/74
C12N15/80 C12N15/82 C12N15/85 C12N15/11 C12N9/12
C12N1/19 C12N1/21 C12N5/10 C07K14/37 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COGONI ET AL.: "Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa" PROC NATL ACAD SCI USA, vol. 94, 16 September 1997 (1997-09-16), pages 10233-10238, XP002136370 ---	
A	COGONI C ET AL: "QUELLING: TRANSGENE-INDUCED GENE SILENCING IN NEUROSPORA CRASSA" NATO ADVANCED SCIENCE INSTITUTES, SERIE H: CELL BIOLOGY,DE,SPRINGER VERLAG, BERLIN, vol. 104, 1998, pages 103-112, XP000906708 ISSN: 1010-8793 --- -/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2000	07/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SHERMAN J M ET AL: "An uncertain silence" TRENDS IN GENETICS,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 13, no. 8, 1 August 1997 (1997-08-01), pages 308-313, XP004084604 ISSN: 0168-9525 ---	
P,X	COGONI : "Neurospora crassa qde-1 gene, partial" EMBL SEQUENCE DATABASE, 10 May 1999 (1999-05-10), XP002144857 HEIDELBERG DE Ac AJ133528 the whole document	1-6, 18-22
P,X	-& COGONI ET AL.: "RNA-dependent RNA polymerase (fragment" EMBL SEQUENCE DATABASE, 1 November 1999 (1999-11-01), XP002144858 HEIDELBERG DE Ac Q9Y7G6 the whole document	1-6, 18-22
P,X	-& COGONI ET AL.: "Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase" NATURE, vol. 399, 13 May 1999 (1999-05-13), pages 166-169, XP002144912 the whole document -----	1-6, 18-22

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* PCT24285

B x No. I TITLE OF INVENTION: ISOLATION AND CHARACTERIZATION OF A *N . CRASSA* SILENCING GENE AND USES TEHREOF.

B x No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA"
L. Aldo Moro 5
00185 ROMA - ITALY

[ ] This person is also inventor

Telephone No. 06/4991.1

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [X] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

MACINO Giuseppe
Dipartimento Biotecnlogie Cellulari ed Ematologia
POLICLINICO UMBERTO I
UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA"
Viale Regina Elena 324
00161 ROMA - ITALY

This person is:

[ ] applicant only

[X] applicant and inventor

[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: [X] agent [ ] common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BANCHETTI Marina - CAPASSO Olga - de SIMONE Domenico - FIORUZZI Maria Augusta - IANNONE Carlo Luigi - TALIERCIO Antonio - ZANARDO Giovanni - ING. BARZANO' & ZANARDO ROMA S.p.A. - Via Piemonte 26 - 00187 ROMA - ITALY

Telephone No. 06/4743241

Facsimile No. 06/4870273

Teleprinter No. 625579

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuati n of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

COGONI Carlo
Dipartimento Biotecnlogie Cellulari ed Ematologia
POLICLINICO UMBERTO I
UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA"
V.le Regina Elena 324
00161 ROMA - ITALY

This person is:

- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked):*

Regional Patent

X	AP	**ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, TZ Tanzania , ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
X	EA	**Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
X	EP	**European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
X	OA	**OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea,GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other king of protection or treatment desired, specify on dotted line)*

National Patent *(if other king of protection or treatment desired, specify on dotted line):*

X	AE	United Arab Emirates		X	LR	Liberia	
X	AL	Albania		X	LS	Lesotho	
X	AM	Armenia		X	LT	Lithuania	
X	AT	Austria		X	LU	Luxembourg	
X	AU	Australia		X	LV	Latvia	
X	AZ	Azerbaijan		X	MD	Republic of Moldova	
X	BA	Bosnia and Herzegovina		X	MG	Madagascar	
X	BB	Barbados		X	MK	The former Yugoslav Republic of Macedonia	
X	BG	Bulgaria		X	MN	Mongolia	
X	BR	Brazil		X	MW	Malawi	
X	BY	Belarus		X	MX	Mexico	
X	CA	Canada		X	NO	Norway	
X	CH and LI	Switzerland and Liechtenstein		X	NZ	New Zealand	
X	CN	China		X	PL	Poland	
X	CU	Cuba		X	PT	Portugal	
X	CZ	Czech Republic		X	RO	Romania	
X	DE	Germany		X	RU	Russian Federation	
X	DK	Denmark		X	SD	Sudan	
X	DM	Dominica		X	SE	Sweden	
X	EE	Estonia		X	SG	Singapore	
X	ES	Spain		X	SI	Slovenia	
X	FI	Finland		X	SK	Slovakia	
X	GB	United Kingdom		X	SL	Sierra Leone	
X	GD	Grenada		X	TJ	Tajikistan	
X	GE	Georgia		X	TM	Turkmenistan	
X	GH	Ghana		X	TR	Turkey	
X	GM	Gambia		X	TT	Trinidad and Tobago	
X	HR	Croatia		X	TZ	Tanzania	
X	HU	Hungary		X	UA	Ukraine	
X	ID	Indonesia		X	UG	Uganda	
X	IL	Israel		X	US	United States of America	
X	IN	India		X	UZ	Uzbekistan	
X	IS	Iceland					
X	JP	Japan		X	VN	Viet Nam	
X	KE	Kenya		X	YU	Yugoslavia	
X	KG	Kyrgyzstan		X	ZA	South Africa	
				X	ZW	Zimbabwe	
X	KP	Democratic People's Republic of Korea		Check-boxes reserved for designating States (for the purposes of			
X	KR	Republic of Korea		a national patent) which have become party to the PCT after			
X	KZ	Kazakhstan		issuance of this sheet:			
X	LC	Saint Lucia					
X	LK	Sri Lanka					

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 22/02/99 22 FEBRUARY 1999	RM99A000117	ITALY		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1)

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Ch ice f International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, ...icate the Authority chosen; the two-letter code may ...used):*
ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(Day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	4
description (excluding sequence listing part)	:	19
claims	:	4
abstract	:	1
drawings	:	5
...quence listing part ...description	:	18
Total number of sheets	:	51

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☒ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify):* STATEMENT

Figure f the drawings which should accompany the abstract: 1

Language of filing of the international application: ENGLISH

B x No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

CAPASSO Olga

For receiving Office use only

1. Date of actual receipt of the purported international application	2. Drawings:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	☐ received:
4. Date of timely receipt of the required corrections under PCT Article 11(2):	☐ not received:
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex t the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference PCT24285

Applicant UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA"

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE — 60.000 [T]

2. SEARCH FEE — 1.829.775 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 51 sheets.

first 30 sheets — 791.934 [b1]

21 remaining sheets x 17.426 additional amount = 365.946 [b2]

Add amounts entered at b1 and b2 and enter total at B — 1.157.880 [B]

Designation Fees

The international application contains _____ designations.

x _____ = 1.363.136 [D]

number of designation fees payable *(maximum 10)* × amount of designation fee

Add amounts entered at B and D and enter total at I — 2.521.016 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* — [P]

5. TOTAL FEES PAYABLE — 4.410.791 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

[ ] The designation fees are not paid at this time.

MODE OF PAYMENT

[ ] authorization to charge deposit account (see below)
[X] bank draft
[ ] coupons
[ ] cheque
[ ] cash
[ ] other *(specify)*:
[ ] postal money order
[ ] revenue stamps

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ _____

[ ] is hereby authorized to charge the total fees indicated above to my deposit account.

[ ] is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

[ ] is hereby authorized to charge the fees for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No. | Date *(day/month/year)* | Signature

See Notes to the fee calculation sheet

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

CAPASSO, Olga
BARZANO & ZANARDO ROMA S.P.A.
26, Via Piemonte
00187 ROMA
ITALIE

FAX 06/4870273

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	19.07.2001
Applicant's or agent's file reference: PCT24285	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT00/00048	16/02/2000	22/02/1999

Applicant
UNIVERSITA DEGLI STUDI DI ROMA "LA SAPIENZA"

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Emslander, S Tel.+49 89 2399-8718

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 November 2000 (02.11.00)	
International application No. PCT/IT00/00048	**Applicant's or agent's file reference** PCT24285
International filing date (day/month/year) 16 February 2000 (16.02.00)	**Priority date** (day/month/year) 22 February 1999 (22.02.99)
Applicant MACINO, Giuseppe et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

19 September 2000 (19.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Zakaria EL KHODARY

Telephone No.: (41-22) 338.83.38

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

BANCHETTI, Marina
Ing. Barzanò & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year) 08 June 2000 (08.06.00)	
Applicant's or agent's file reference PCT24285	**IMPORTANT NOTIFICATION**
International application No. PCT/IT00/00048	International filing date (day/month/year) 16 February 2000 (16.02.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 22 February 1999 (22.02.99)
Applicant UNIVERSITÁ DEGLI STUDI DI ROMA "LA SAPIENZA" et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
22 Febr 1999 (22.02.99)	RM99A000117	IT	07 June 2000 (07.06.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Zakaria EL KHODARY

Telephone No. (41-22) 338.83.38

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
CAPASSO, Olga
BARZANO & ZANARDO ROMA S.P.A.
26, Via Piemonte
00187 ROMA
ITALIE

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*: 09. 10. 00

Applicant's or agent's file reference: PCT24285

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/ IT 00/ 00048	16/02/2000	22/02/1999

Applicant: UNIVERSITA DEGLI STUDI DI ROMA "LA SAPIENZA"

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

19/09/2000

2. This date of receipt is:

- [x] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

[ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/
European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0, Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer
KAUFMANN S B
Tel. (+49-89) 2399-2615

EUROPÄISCHES PATENTAMT - EUROPEAN PATENT OFFICE - OFFICE EUROPÉEN DES BREVETS

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

T :
BANCHETTI, Marina
Ing. Barzanò & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year)	
31 August 2000 (31.08.00)	
Applicant's or agent's file reference PCT24285	**IMPORTANT NOTICE**
International application No. PCT/IT00/00048	**International filing date** (day/month/year) 16 February 2000 (16.02.00)
Priority date (day/month/year) 22 February 1999 (22.02.99)	
Applicant UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA" et al	

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE, GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ, OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   31 August 2000 (31.08.00) under No. WO 00/50581

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
BARZANO & ZANARDO ROMA S.P.A.
Attn. BANCHETTI, Marina
26, Via Piemonte
00187 ROMA
ITALY

Applicant's or agent's file reference PCT24285	Date of mailing *(day/month/year)* 07/09/2000 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/IT 00/ 00048	International filing date *(day/month/year)* 16/02/2000
Applicant UNIVERSITA DEGLI STUDI DI ROMA "LA SAPIENZA"	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Chantal Meyer

Form PCT/ISA/220 (July 1998)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) th claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the internati nal application is English, the l tter must be in English; if the language f the international application l French, the letter must be in French.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/00

A2

(11) International Publication Number: **WO 00/50581**

(43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/IT00/00048

(22) International Filing Date: 16 February 2000 (16.02.00)

(30) Priority Data:
RM99A000117 22 February 1999 (22.02.99) IT

(71) Applicant *(for all designated States except US)*: UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA" [IT/IT]; P.le Aldo Moro, 5, I–00185 Roma (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MACINO, Giuseppe [IT/IT]; Policlinico Umberto I, Università Degli Studi Di Roma "La Sapienza", Dipartimento Biotecnologie Cellulari ed Ematologia, V.le Regina Elena, 324, I–00161 Roma (IT). COGONI, Carlo [IT/IT]; Policlinico Umberto I, Università Degli Studi Di Roma "La Sapienza", Dipartimento Biotecnologie Cellulari ed Ematologia, V.le Regina Elena, 324, I–00161 Roma (IT).

(74) Agents: BANCHETTI, Marina et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I–00187 Roma (IT).

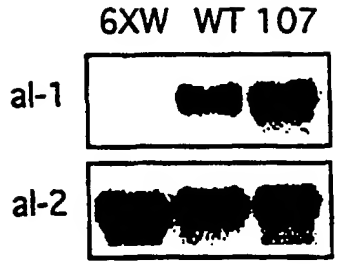
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

(57) Abstract

A nucleotide sequence encoding for a protein characterized in that it has a silencing activity and comprises a RNA–dependent RNA polymerase domain is disclosed; furthermore expression vectors suitable for the expression of said sequence in bacteria, plants, animals and fungi are disclosed; the invention refers also to organisms transformed by such vectors.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
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BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
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BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
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BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
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ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

The present invention relates to the isolation and characterization of a *Neurospora crassa* gene encoding for an essential activity in the co-suppression process and to uses and applications thereof in vegetal, animal and fungine fields.

The production of transgenic organisms is of large utility both in basic and applied biological research. The transgenic DNA is usually integrated in the genome and transferred as a Mendelian character. However, in various instances, the transgene introduction induces gene silencing phenomena (Flavell, R.B. 1994), i.e. the repression of the expression of the transgene itself and/or of one or more endogenous homologous genes.

The gene silencing can act at two levels: transcriptional (trans-inactivation) where transgenes contain sequences homologous to the silenced gene promoter (Vaucheret, 1993); and post-transcriptional (co-suppression) which requires homologies between coding regions (Flavell, 1994; Stam et al., 1997; Baulcombe, 1996).

Generally the silencing induced by a transgene requires an almost complete sequence homology (from 70% to 100%) between transgene and silenced gene sequences (Elkind, 1990).

In the *Neurospora crassa* filamentous fungus, during the vegetative phase, the presence of transgenes induces a post-transcriptional gene silencing phenomenon, named "quelling" (Cogoni et al., 1996).

By using the *al-1* gene (albino 1) (Schmidhauser et al., 1990) as silencing visual marker, many features of the phenomenon have been discovered (Cogoni et al., 1996). Particularly the *al-1* gene "quelling" in *Neurospora* is characterized in that: 1) the gene silencing is reversible further to the loss of transgene copies; 2) the reduction of mRNA basal level results from a post-transcriptional effect; 3) transgenes containing at least a region of 132 base pairs which is identical to the region encoding for the target gene are sufficient to induce the "quelling"; 4) the duplication of promoter sequences is ineffective to induce the silencing; 5) the "quelling" exhibits a dominant behavior in eterocarions containing both transgenic and untransformed nuclei, indicating the involvement of a molecule which is acts "in trans" among the nuclei; 6) the expression of an aberrant RNA transcribed by the transgenic locus is strictly correlated to silencing, suggesting that the "quelling" can be induced and/or mediated by a transgenic RNA molecule.

Therefore homologies between *Neurospora* silencing and plant co-suppression can be pointed out. The gene silencing in *Neurospora* is reversible, as result of transgenic copies instability during mitotic phase; in plants also the co-suppression reversion is associated with the reduction of transgene copy number, resulting from intra-chromosomal recombination during mitosis or meiosis (Mittelstein Scheid et al., 1994; Stam et al., 1997). Thus both in plants and in *Neurospora* the transgene presence is required to maintain the silencing. As in *Neurospora,* a decrease of the mRNA basal level of the silenced gene results from a post-transcriptional

mechanism (Dehio and Schell 1994; van Blokand et al., 1994; de Carvalho et al., 1995). Furthermore to induce the "quelling", transgenes must contain a portion of the silencing target gene coding sequence, being the promoter region ineffective. In plants coding regions with no promoter sequences can induce silencing (van Blokand et al., 1994) and, as in the "quelling", promoters or functionally active gene products are not required for the co-suppression.

One of the similarities between "quelling" and co-suppression in plants is that both mechanisms are mediated by diffusion factors. In *Neurospora* eterokaryotic strains, nuclei wherein the *albino-1* gene is silenced are able to induce the *al-1* gene silencing of the other not transformed nuclei, all sharing the same cytoplasmic environment (Cogoni et al., 1996). In plants the presence of a diffusion factor results from the fact that the co-suppression is effective in inhibiting the replication of Tobacco Etch Virus (TEV), a RNA virus with an exclusively cytoplasmic cycle. The occurrence of highly diffusible factors, which are effective to mediate the co-suppression, has been demonstrated using the grafting technique in tobacco (Palaqui et al., 1997), showing that silenced tobacco plants are able to transfer the silencing to non-silenced plants through grafting.

The fact that "quelling" and co-suppression share all these features suggests that mechanisms involved in post-transcriptional gene silencing in plants and in fungi can be evolved by an ancestral common mechanism.

Recently gene inactivation phenomena resulting from transgene introduction have been disclosed in animals. In *Drosophila melanogaster* the location of a transgene close

to heterochromatic centers results in a variegate expression (Wallrath and Elgin, 1995; Pirrotta, V., 1997). Similar expression profiles have been observed when the reference transgene is within tandem arrayed transposons, indicating that tandem repeats are effective to induce the chromatin condensation. (Dorer and Henikoff, 1994). Again in *Drosophila* Pal-Bhadra et al. (1997) have observed that the transgene introduction can lead to gene inactivation phenomena, similar to the co-suppression.

Gene silencing phenomena resulting from transegene sequence repeats have been disclosed recently in mammalians.

Garrick et al. (1998) produced mouse transgenic lines wherein 100 transgenic copies are present only in a locus and are directly tandem arrayed. The transgene expression has been disclosed to be inversely proportional to the number of occurring copies, indicating that silencing phenomena dependent on repeat copies are present also in mammalians.

Therefore the identification of *Neurospora* genes which are involved in the silencing is the first step to modulate the same process in plants, animals and fungi. The silencing modulation is of great relevance when transgenic organisms able to express the desired phenotype are produced.

The authors of the present invention have already isolated *Neurospora crassa* strains having mutations regarding essential functions for gene silencing mechanism (Cogoni and Macino, 1997); 15 independent isolated mutants define three complementation groups, thus identifying the *qde-1, qde-2 and qde-3* genes (*qde*

stands for "quelling"-deficient), whose products are essential to the silencing machinery. *qde* genes are essential to the *Neurospora* silencing, as suggested by the fact that silencing of three independent genes (*al-1*, *al-2* and *qa-2*) is impaired by *qde* mutations (Cogoni and Macino, 1997).

The authors of the invention have identified and cloned now one out of *Neurospora qde* genes, the *qde-1* gene, thus identifying one of required factors for silencing. By considering the similarity between "quelling" and co-suppression, genes orthologous to the isolated gene are involved in co-suppression and more generally in gene silencing in other organisms, like plants, fungi and animals.

The present invention can be applied with reference to two general scope: 1) silencing potentiation as a tool for inactivating more effectively and durably a desired gene, and 2) silencing suppression to obtain a better expression of the introduced transgenes.

As to the silencing potentiation, the over-expression of one or more genes controlling the phenomenon can lead to higher efficiency and/or stability thereof. Therefore the introduction of *qde-1* gene or of homologous genes thereof in microorganisms can constitute a tool to repress more effectively gene functions. Particularly this approach is specially useful in plants wherein the co-suppression is usually used for the "knock-out" of gene functions. In plants again the gene silencing potentiation can be used to obtain lines resistant to pathogen virus, by introducing transgenes encoding for viral sequences, in order to achieve the

expression inhibition of the virus itself (Flavell et al., 1994).

Analogous applications are suitable for animals, wherein some indications suggest that silencing can inhibit the suitable expression of introduced transgenes (Garrick et al., 1998).

On the contrary, there are instances wherein it is desirable not to have or to reduce the gene silencing, i.e. where a transgene is to be over-expressed. It is known that the co-suppression is strictly correlated both with the presence of an high copy number of the transgene, and with a transgene high expression. This correlation can hamper the production of transgenic organisms which express a transgene at high levels, because more high is the expression and/or the copy number, more probable is to evoke silencing responses. As above mentioned, analogous mechanisms of gene inactivation, dependent on a high copy number, have been disclosed in animals. In these circumstances plant or animal lines, totally or partially ineffective for silencing, constitute an ideal recipient wherein the desired gene can be over-expressed. The invention can be applied within this scope using different approaches:

A) Identification and production of mutant lines in genes homologous to *qde-1* gene, in plants, animals and fungi.

The knowledge of *Neurospora qde-1* gene, essential for silencing mechanism, can allow the isolation of mutant lines in other organisms, mutated in genes homologous to *qde-1*. For example by means of amplifications using degenerated primers, designed from the most conserved regions of *qde-1* gene, mutant lines in

homologous genes can be identified, by analysis of insertion mutant gene banks, already available for many plant species. Both in fungi and animals such mutants can be obtained, following the identification of the homologous gene, by means of "gene disruption" techniques using homologous recombination.

B) Reduction of *qde-1* gene expression

Other strategies for the production of silencing-deficient lines comprise the use of *Neurospora qde-1* gene or homologous genes thereof. *qde-1* or homologous genes can be introduced into suitable expression vectors to express them in an anti-sense orientation in order to inhibit the expression of resident endogenous genes. Alternatively portions of *qde-1* or of homologous genes can be over-expressed, in order to obtain a negative dominant effect and thus blocking the function of *qde-1* endogenous genes.

The authors of the present invention have cloned and characterised the *Neurospora crassa qde-1 gene*. The sequence analysis of the *qde-1* gene detected a region having a significant homology with a RNA-dependent RNA polymerase, isolated from tomato, which was suggested, but not demonstrated, to be involved in the co-suppression mechanism (Schiebel et al., 1998).

The authors of the invention for the first time have demonstrated that a gene encoding for a RNA-dependent RNA polymerase is involved in gene silencing induced by transgenes. Therefore for the first time it is disclosed that a gene belonging to the RNA-dependent RNA polymerase family is an essential component also for inactivation mechanism of the repeat sequences.

Within the scope of the invention the reference to homology per cent means similarity per cent, i.e. number of identical residues + number of conserved residues with respect to the total residues of the considered sequence.

Therefore it an object of the present invention a nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA dependent RNA polymerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Preferably the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. More preferably the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Most preferably the domain comprises the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. According to a particular embodiment the nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof. Even more preferably the nucleotide sequence is the nucleotide sequence of SEQ ID No. 1 or its complementary sequence.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in bacteria, the nucleotide sequence of the invention. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the expression in bacteria can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in plants or in specific plant organs, the

nucleotide sequence of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the invention in plants or in specific plant organs can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in fungi, the nucleotide sequence of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the inventive protein in fungi can be used and it is within the scope of the invention.

A further object of the invention is an expression vector comprising, under the control of a promoter which is expressed in animals, the nucleotide sequence of the invention, both in a sense and anti-sense orientation. Those skilled in the art will appreciate that any plasmid suitable for a correct and effective expression of the protein of the invention in animals can be used and it is within the scope of the invention.

A further object of the invention is a prokaryotic organism transformed by using the expression vector active in bacteria of the invention.

A further object of the invention is a plant or a specific plant organ transformed by using the expression vector active in plants of the invention.

A further object of the invention is a plant mutated at the nucleotide sequence of the invention having a reduced or inhibited silencing activity.

A further object of the invention is a fungus transformed with the expression vector of the invention active in fungi.

A further object of the invention is a fungus mutated at the nucleotide sequence of the invention and having reduced or inhibited silencing activity.

A further object of the invention is a non-human animal transformed with the expression vector of the invention active in animals.

A further object of the invention is a non-human animal mutated at the nucleotide sequence of the invention and having a reduced or inhibited silencing activity.

A further object of the invention is a not human animal mutated at the nucleotide sequence of the invention and having reduced or inhibited silencing activity.

A further object of the invention refers to a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polimerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Preferably the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. More preferably the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. Most preferably the domain comprises the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1. According to a particular embodiment the nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

It is within the scope of the present invention the use of the nucleotide sequence of the invention to modulate gene silencing in plants, animals and fungi.

The present invention now will be disclosed by way of non limiting examples with reference to the following figures:

Figure 1 shows the restoration of the *al-1* expression in 107 insertional mutant strain. The total RNA has been extracted from mycetes collected after light induction over ten minutes from an *al-1* silenced strain (6XW), a untransformed wild type strain (WT) and 107 mutant strain. For the hybridization an *al-1* specific probe was used. In the lower part the restoration using an *al-1* specific probe is showed.

Figure 2 shows the genomic organization of the *qde-1* gene. a) The two cosmides (56G11 and 40H7) able to complement the *qde-1* mutants are represented. The white box in the 40H7 cosmid represents the sequences of the cosmid vector. A restriction map of 7,9 Kb *qde-1* containing fragment obtained from 40H7 using EcoRi is showed: E(EcoRI), P(PstI), B(BgIII). The black box represents the ORF identified within EcoRi 7,9 Kb fragment. The pDX and pSX plasmids containing the DNA fragments subcloned in the XbaI (X) and EcoRI (E) sites are also showed. B) Southern analysis of the 107 and WT strains. The genomic DNA was digested using BgII and NaeI. In the lower diagram the DNA probe used for the hybridization and the expected BgII/NaeI(B/N) restriction fragments are reported. The triangle represents the integration site in the 107 strain which determines the disappearance of the 1,0 Kb restriction fragment.

Figure 3 represents the expression of the *qde-1* gene in the 107 insertional mutant strain, untransformed wild type (WT) strain and al-1 silenced strain (6XW). The total RNA was hybridizated using a *qde-1* specific probe. In the lower part the amount of gel loaded RNA is showed.

Figure 4 represents the amino acid sequence deduced from the *qde-1* gene. The underlining indicates the RdRP conserved domain as showed in the alignment of Figure 5.

Figure 5 represents a sequence alignment of the QDE-1 protein with other polypeptides from SwissProtein sequence database: ORF from Z488334 (elegl) C. *elegans*, ORF from Z98533 (pom) *S. pombe*, ORF from AF080120 (araB) *A. thaliana* and RNA-dependent RNA polymerase from Y104403 (RdRP) tomato. Identical residues are pointed out in black, whereas the conservative replacements are showed in gray.

Materials and Methods

Strains, growing and transforming conditions

The methodology and heterokaryon analysis in *Neurospora crassa* substantially was the same as described in (Davis and De Sevres, 1970). The spheroplasts are prepared according to method of Vollmer and Yasnofsky (1997). The 107 strain was isolated in the following way: a *qde-1* silenced strain, called 6xw, already described (Cogoni and Macino, 1997), was transformed with pMXY2 which contains the benomyl resistant beta-tubulin gene, which acts as dominant selectable marker in *N. Crassa* (Staben et al. 1989). Transformed strains able to grow in the presence of benilate containing medium were selected on the base of the carotenoid biosynthesis by visualization of the conidium colors: the conidia from the wild type strains were bright orange, whereas those

from transformed strains having colors from white to yellow were indicative of a silencing activity.

Plasmids and gene libraries

The genomic gene *qde-1* was isolated from a *N. Crassa* gene library in cosmides (Cabibbo et al., 1991). The sub-cloning of the restriction fragments from the gene library clones was carried out in the pBSK plasmid. Therefore the sub-clones were used in co-transforming experiments using pMXY2 or pES200 (containing the hygromycin resistant gene).

Southern and Northern Hybridizations

Chromosomal DNA was prepared according to Morelli et al. (1993). After digestion, the genomic DNA was transferred according to Maniatis et al. (1982). The probes were labeled by casual priming (Boheringer). The RNA was electrophoresed on agarose gel, transferred and blotted on Hybond N membranes.

DNA Analysis and Sequencing

The *qde-1* nucleotide sequence was determined for both strands using TAQ FS polymerase and the fluorescence method and analyzed using an Applied Biosystems 373A automated apparatus; the nucleotide and amino acid derived sequences were analyzed by means of MacMolly Tetra program. A protein comparison was carried out using the BLASTP method. The ClustaIW algorithm was used for the alignment.

Results

In order to clone the *qde* genes an insertional mutagenesis on an *al-1* transgenic strain (6XW) which shows an albino phenotype (white) resulting from a post-transcriptional silencing of the *al-1* endogenous gene was used: out of 100.000 independent transformed insertional

strains, a strain (107) showed a reversion of the gene silencing visible as restoration of a bright orange wild type phenotype. The bright orange wild type phenotype of the 107 strain results from the restoration of the expression of *al-1* mRNA, as demonstrated by a Northern analysis (see Figure 1). Furthermore an heterokaryon assay revealed the mutation to be recessive and *trans* acting. In addition by means of the heterokaryon assay it was possible to establish that the 107 strain mutant belongs to one of the three already identified complementation *qde* groups (Cogoni and Macino 1997). The restoration of an *al-1* silenced phenotype occurs in heterokaryons with *qde-2* and *qde-3* mutants. It is not possible to complement with *qde-1* mutants (Table 1), indicating that the 107 strain is mutated at the *qde-1* gene.

Table 1 - The 107 strain is mutated at the *qde-1* gene

qde mutant strains used in specific heterokaryons

	107	M17	M18	M10	M11	M7	M20
107	WT	AL	AL	AL	AL	WT	WT
M17		WT	WT	AL	AL	AL	AL
M18			WT	AL	AL	AL	AL
M10				WT	WT	AL	AL
M11					WT	AL	AL
M7						WT	WT
M20							WT

WT = heterocaryon with a wild type phenotype for carotenoid (bright orange);

AL = heterocaryon with an albino phenotype wherein the *al-1* gene silencing is restored.

The *qde* mutant strains were described by Copgoni and Macino (1997); M17 and M18 are *qde-3* mutants; M10 and

M11 are *qde-2* mutants; M7 and M20 are *qde-1* mutants. The heterokaryon phenotypes were examined by visual inspection after a seven day growth in the presence of light.

In order to isolate the *qde-1 gene,* the "tagging" plasmid was recovered from the 107 strain by means of a procedure suitable for the liberation of the plasmid using the *BglII* restriction enzyme having a single restriction site within the tagging plasmid. The pCR4 plasmid was recovered after chromosomal DNA re-ligation following a *Bgl*II restriction (see figure 2a). The chromosomal DNA flanking the insertion site was isolated using the *BglII* and *PstI* enzymes and the resultant restriction fragment was used to probe a *N. crassa* genomic library in cosmids. Cosmids 58G11 and 40H7, both positively hybridizating, were introduced by means of an UV induced transforming experiment in the 107 strain and in the M7 qde-1 mutant strain. Both the cosmids were able to complement the two assayed *qde-1* mutants. The restoration of the *al-1* gene silencing which determines the appearance of a white phenotype indicates that both the cosmids contain a functional *qde-1* gene. By using the same flanking DNA like a probe, two EcoRi fragments from 40H7 and 58G11, respectively having 7,9 and 10,0 kB, were identified and subcloned (see figure 2a). Both the EcoRi fragments (p79E and p10E plasmids) are able to complement the *qde-1* strain (107 and M7) but not the *qde-2* (M10) and *qde-3* (M17) mutant strains (Table 2), indicating that the *qde-1* functional gene is contained in the 7,9 Kp EcoRi fragment.

Table 2 Complementation with the *qde-1* gene

Plasmids	qde mutant strains used			
	107	M7(qde-1$^-$)	M10(qde-2$^-$)	M17(qde-3$^-$)
P79E	58/200(29%)	48/200(24%)	0/200(0%)	0/200(0%)
P10E	51/100(25%)	25/100(25%)	0/200(0%)	0/200(0%)
PSX	0/200(0%)	0/200(0%)	-	-
PDX	0/200(0%)	0/200(0%)	0/200(0%)	0/200(0%)

The complementation frequency is reported as per cent of the transforming strains which show an albino phenotype with respect to the total number of transforming strains. The pMXY2 plasmid was used as negative control.

The ability of the *qde-1* gene in restoring the *al-1* gene silencing only in corresponding mutants, excludes the possibility that the DNA cloned fragment is able to restore the *al-1* gene silencing, apart from the *qde* complementation group. In addition the M7 strain transformed with the 7,9 Kb EcoRI fragment allows the ability in silencing an other *al-2* carotenogenic gene when introduced by transformation (not showed data). The 7,9 Kb EcoRI fragment was further cloned using the XbaI site (see figure 2a) which cuts the center the EcoRI fragment. Both the XbaI/EcoRI (pSX and pDX plasmids) fragments were not able to complement the 107 and M7 strains (see Table 2), suggesting that the XbaI site is probably localised within the *qde-1* gene.

The whole region of the EcoRI fragment which includes the putative *qde-1* gene and the adjacent regions were sequenced, revealing an open reading frame (ORF) of 4206 bp which encodes for a putative protein containing 1402 amino acids. Two different results suggest that this ORF corresponds to the *qde-1* gene. Firstly the XbaI

restriction site used to subclone the 7,9 Kb EcoRI fragment is localized in the center of the ORF (figure 2a) and therefore is consistent with the result that both the KbaI/EcoRI fragments are not able to complement the *qde-1* mutation (Table 2). Secondly by means of Southern analysis (see Figure 2b) the insertion site of the tagging plasmid in the 107 strain was mapped to be within the BglII and NaeI restriction sites in the ORF. No size variation of the flanking regions was detected, therefore excluding the possibility that the deletions include other ORFs within the 7,9 Kb EcoRi region. The cDNAs synthesized by inverted PCR (RT-PCR) revealed a co-linearity with the genomic DNA indicating that no intron is present. The expression of the *qde-1* gene was analyzed by Northern analysis using a probe including the *qde-1* ORF (figure 2a). Thus a transcript of about 5000 nt was detected and further it was found out that the *qde-1* mRNA basal level in an *al-1* silenced strain (6XW) was twice than in a not transformed WT strain (see figure 3). In addition the *qde-1* whole length mRNA is not detectable in the *qde-1* 107 insertional mutant strain where, on the contrary, a smaller band is included suggesting that the *qde-1* truncated transcripts are produced as the integration result. The fact that the *qde-1* gene expression is specifically increased in a silenced strain suggests the existence of a regulatory mechanism able to activate cell components of the silencing machinery in the transgenic strains.

The QDE-1 protein deduced from the nucleotide sequence contains 1402 amino acids (see figure 4), the molecular weight and statistical pI thereof being 158.004 Da and 8.0, respectively. The QDE-1 protein does not

contain a signal peptide or a transmembrane domain indicating that it is probably an intracellular protein. Furthermore the idiopathic plot suggests that QDE-1 is a soluble protein. A BLAST study showed that *qde-1* has an homology statistically significant with hypothetical proteins from various other organisms comprising: two ORFs from *C. elegans* (Z4834 and Z78419 EMBL entry numbers) with expected values (E value) of 2e-16 and 9e-10, respectively, one ORF from *S. pombe* (Z98553 EMBL entry number) with an 3e-13 E value; four ORFs from *A. thaliana* (AF080120 and AC005169 EMBL entry numbers, the latter comprising thre ORFs at the same chromosomal localization,) and 8e-15, 7e-06, 4e-05, 5e-02 E values, respectively. Finally a significant homology (2e-17 E value) with a putative protein coded by tomato cDNA (Y10403 EMBL entry number) was discovered. The discovered homology does not extend over the whole protein but it is limited to a portion containing 570 amino acids, from aa. 710 to aa. 1282, which defines a conserved domain (see figure 5). Among the identified putative homologous proteins only that derived from the sequence of tomato cDNA was functionally characterized as a RNA-dependent RNA polymerase (RdRP, 9).

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CLAIMS

1. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain, wherein the domain is at least 30% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

2. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 1, wherein the domain is at least 40% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

3. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 2, wherein the domain is at least 50% homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

4. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 3, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

5. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 4, wherein said nucleotide sequence encodes for a protein having the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

6. Nucleotide sequence encoding for a protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain

according to claim 4, wherein said nucleotide sequence is the sequence of SEQ ID No. 1 or its complementary sequence.

7. Expression vector comprising, under the control of a promoter that is expressed in bacteria, the nucleotide sequence according to any one of claims 1-6.

8. Expression vector comprising, under the control of a promoter that is expressed in plants or in specific plant organs, the nucleotide sequence according to any one of claims 1-6 in a sense and anti-sense orientation.

9. Expression vector comprising, under the control of a promoter that is expressed in fungi, the nucleotide sequence according to any one of claims 1-6 in a sense and anti-sense orientation.

10. Expression vector comprising, under the control of a promoter that is expressed in animals, the nucleotide sequence according to any one of claims 1-6 in a sense and anti-sense orientation.

11. Prokaryotic organism transformed by using the expression vector active in bacteria according to claim 7.

12. Plants or a specific plant organ transformed by using the expression vector active in plants according to claim 8.

13. Plant mutated at the nucleotide sequence according to any one of claims 1-6 having a reduced or inhibited silencing activity.

14. Fungus transformed by using the expression vector active in fungi according to claim 9.

15. Fungus mutated at the nucleotide sequence according to any one of claims 1-6 having a reduced or inhibited silencing activity.

16. Non-human animal transformed by using the expression vector active in animals according to claim 10.

17. Non-human animal mutated at the nucleotide sequence according to any one of claims 1-6 having a reduced or inhibited silencing activity.

18. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain wherein the domain is at least 30 % homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

19. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 18, wherein the domain is at least 40 % homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1

20. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 19, wherein the domain is at least 50 % homologous with the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1

21. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 20, wherein the domain is the amino acid sequence from aa. 710 to aa. 1282 of SEQ ID No. 1.

22. Protein characterized in having a silencing activity and in comprising a RNA-dependent RNA polymerase domain according to claim 21 comprising the amino acid sequence of SEQ ID No. 1 or functional portions thereof.

23. Use of the nucleotide sequence according to any one of claims 1-6 to modulate the gene silencing in plants, animals and fungi.

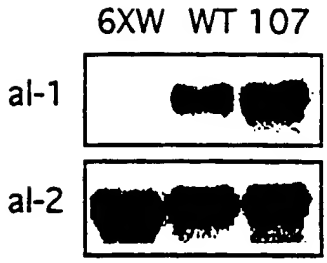
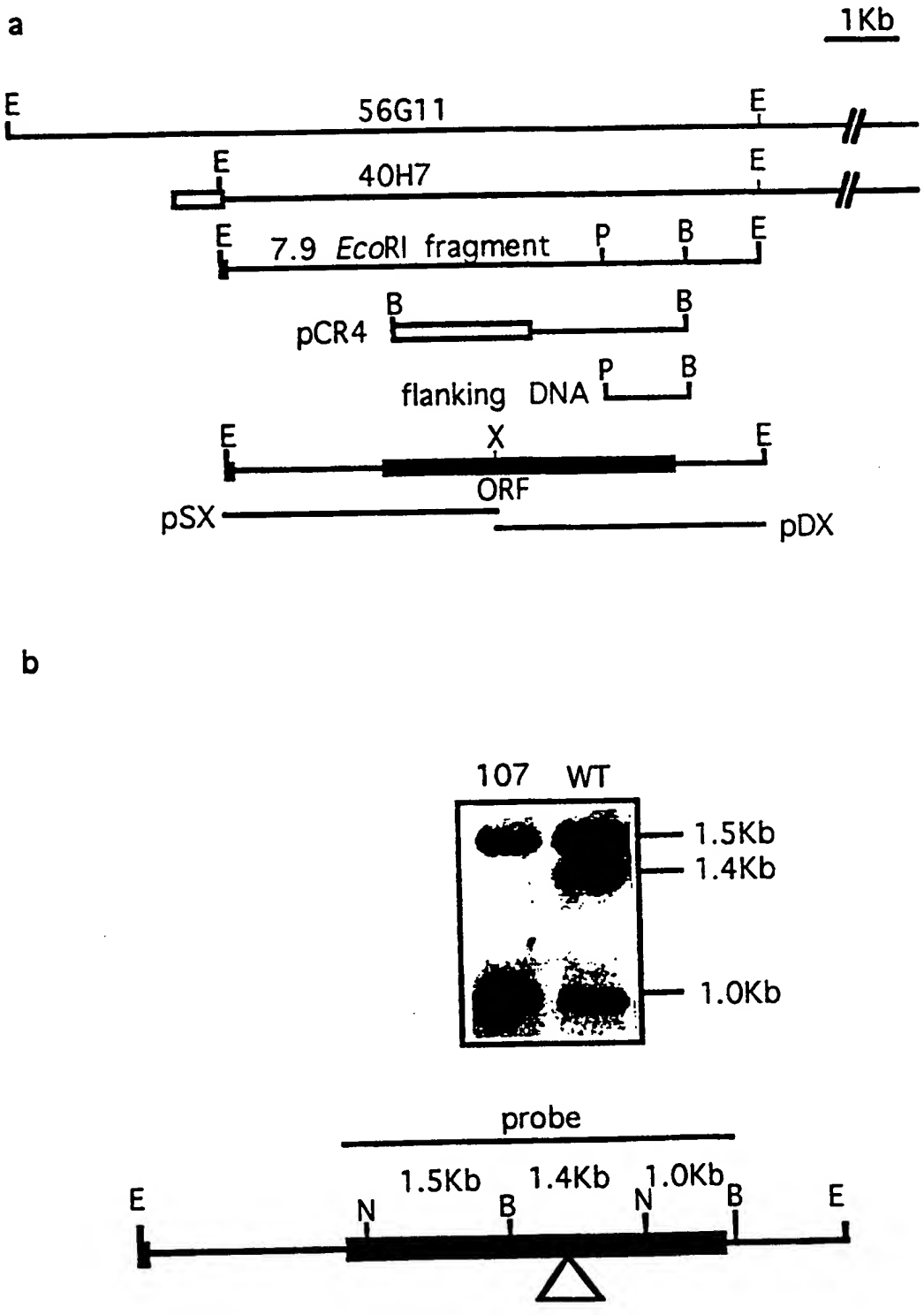


FIG. 1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flanking DNA
E
X
E
ORF
pSX
pDX
b
107
WT
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FIG. 2

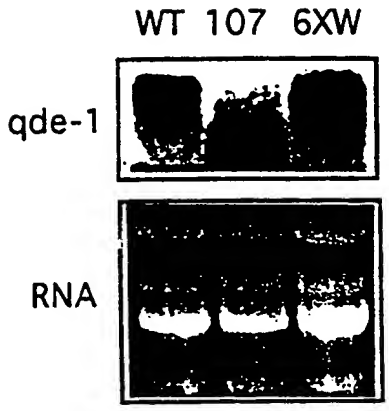

FIG. 3

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TVLLEVLNRF MPPPNNTPGR TFGRTLSGPS GLSRPTSTNT KRKDEPANVT FADPPKRSLT    180
RSATGPPIHG AAIPLKFPDP VNTGSKRPSL ESENLNQCTK RAKGKLSDNV AAAAAPPVPI    240
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APPSQPREKR PVDATVFEAG HLIESPSKGR TTKSHIDNQP LSSSSQGETS FSTYYESFPS    360
SGGEGAIPEP SRSNGLARSE ESARSQVQVH APVVAARLRN IWPKFPKWLH EAPLAVAWEV    420
TRLFMHCKVD LEDESLGLKY DPSWSTARDV TDIWKTLYRL DAFRGKPFPE KPPNDVFVTA    480
MTGNFESKGS AVVLSAVLDY NPDNSPTAPL YLVKLKPLMF EQGCRLTRRF GPDRFFEILI    540
PSPTSTSPSV PPVVSKQPGA VEEVIQWLTM GQHSLVGRQW RAFFAKDAGY RKPLREFQLR    600
AEDPKPIIKE RVHFFAETGI TFRPDVFKTR SVVPAEEPVE QRTEFKVSQM LDWLLQLDNN    660
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RIRDVLGLGD VPSAVQGRFG SAKGMWVIDV DDTGDEDWIE TYPSQRKWEC DFVDKHQRTL    780
EVRSVASELK SAGLNLQLLP VLEDRARDKV KMRQAIGDRL INDLQRQFSE QKHALNRPVE    840
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FIG. 4

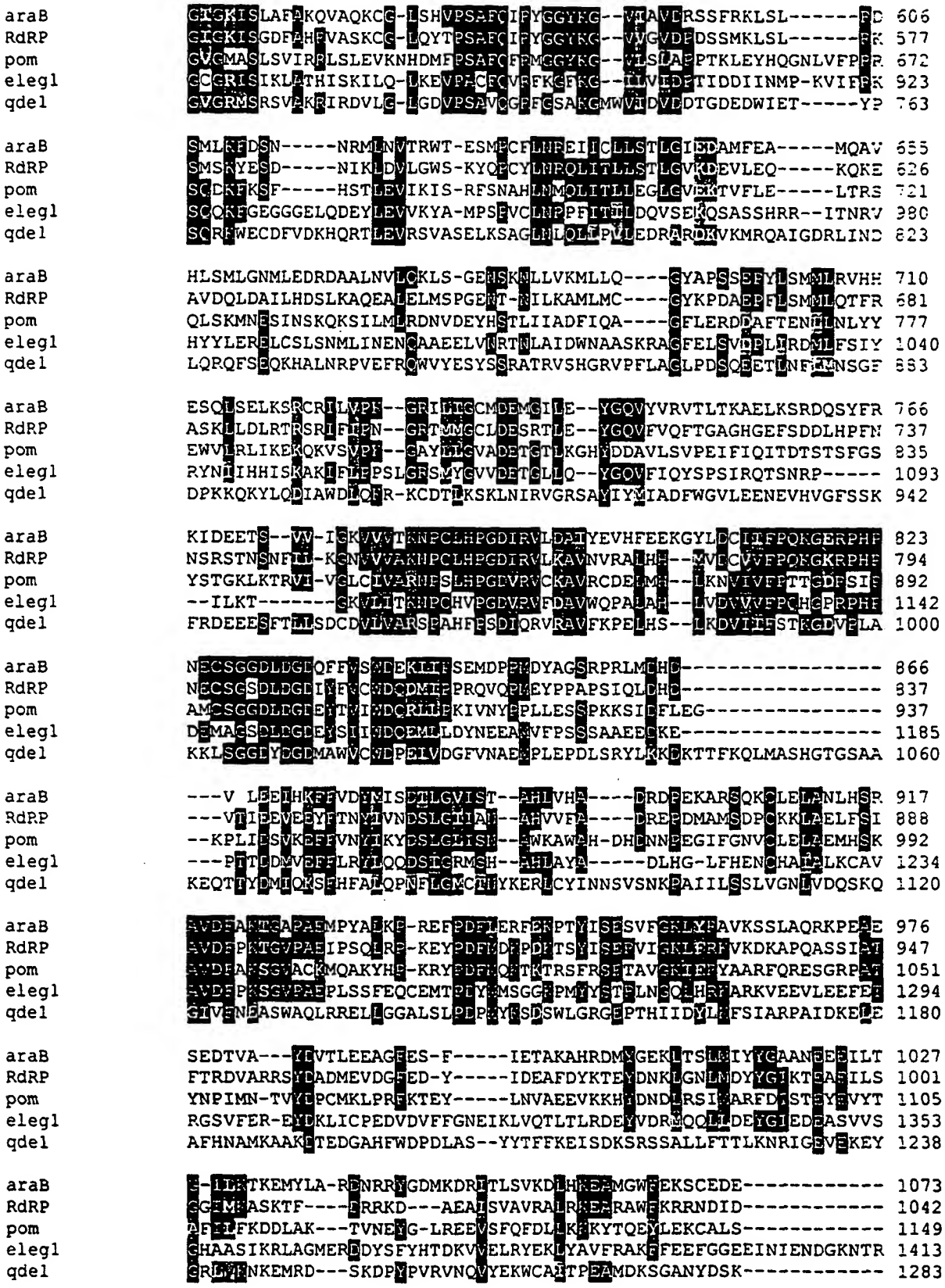

FIG. 5

SEQUENCE LISTING

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Cogoni Carlo
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Lys Ala Ala Ser Ser Asn Trp Val Pro Lys Ala His Ala Asp Pro Asp
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Phe Pro Glu Lys Pro Pro Asn Asp Val Phe Val Thr Ala Met Thr Gly
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Asn Phe Glu Ser Lys Gly Ser Ala Val Val Leu Ser Ala Val Leu Asp
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Tyr Asn Pro Asp Asn Ser Pro Thr Ala Pro Leu Tyr Leu Val Lys Leu
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                520                 525                 530

cct gat agg ttt ttc gag atc ctt ata ccg tcg cct acg agc acc agc   4087
Pro Asp Arg Phe Phe Glu Ile Leu Ile Pro Ser Pro Thr Ser Thr Ser
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Pro Ser Val Pro Pro Val Val Ser Lys Gln Pro Gly Ala Val Glu Glu
        550                 555                 560

gtc atc cag tgg ctc acg atg ggg caa cat tct ctt gta ggc cgc caa   4183
Val Ile Gln Trp Leu Thr Met Gly Gln His Ser Leu Val Gly Arg Gln
    565                 570                 575

tgg cgc gct ttc ttc gcc aaa gat gcc gga tac agg aaa cct ctc agg   4231
Trp Arg Ala Phe Phe Ala Lys Asp Ala Gly Tyr Arg Lys Pro Leu Arg
580                 585                 590                 595
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Val His Phe Phe Ala Glu Thr Gly Ile Thr Phe Arg Pro Asp Val Phe
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        630                 635                 640

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Glu Phe Lys Val Ser Gln Met Leu Asp Trp Leu Leu Gln Leu Asp Asn
    645                 650                 655

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Asn Thr Trp Gln Pro His Leu Lys Leu Phe Ser Arg Ile Gln Leu Gly
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                680                 685                 690

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His His Lys Thr Asp Leu Leu Ser Pro Ser Gly Thr Gly Glu Val Met
            695                 700                 705

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 August 2000 (31.08.2000)

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WO 00/50581 A3

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(21) International Application Number: PCT/IT00/00048

(22) International Filing Date: 16 February 2000 (16.02.2000)

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(30) Priority Data:
RM99A000117 22 February 1999 (22.02.1999) IT

(71) Applicant *(for all designated States except US)*: **UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA"** [IT/IT]; P.le Aldo Moro, 5, I-00185 Roma (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MACINO, Giuseppe** [IT/IT]; Policlinico Umberto I, Università Degli Studi Di Roma "La Sapienza", Dipartimento Biotecnologie Cellulari ed Ematologia, V.le Regina Elena, 324, I-00161 Roma (IT). **COGONI, Carlo** [IT/IT]; Policlinico Umberto I, Università Degli Studi Di Roma "La Sapienza", Dipartimento Biotecnologie Cellulari ed Ematologia, V.le Regina Elena, 324, I-00161 Roma (IT).

(74) Agents: **BANCHETTI, Marina** et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

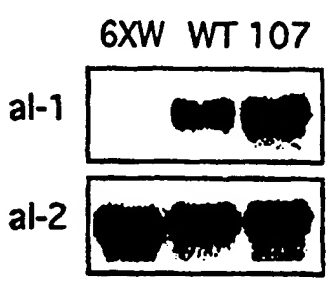
Published:
— *With international search report.*

(88) Date of publication of the international search report:
30 November 2000

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ISOLATION AND CHARACTERIZATION OF A *N. CRASSA* SILENCING GENE AND USES THEREOF

(57) Abstract: A nucleotide sequence encoding for a protein characterized in that it has a silencing activity and comprises a RNA-dependent RNA polymerase domain is disclosed; furthermore expression vectors suitable for the expression of said sequence in bacteria, plants, animals and fungi are disclosed; the invention refers also to organisms transformed by such vectors.



WO 00/50581 A3

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/IT 00/00048

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N15/63 C12N15/67 C12N15/70 C12N15/74
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
STRAND, EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COGONI ET AL.: "Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa" PROC NATL ACAD SCI USA, vol. 94, 16 September 1997 (1997-09-16), pages 10233-10238, XP002136370 ---	
A	COGONI C ET AL: "QUELLING: TRANSGENE-INDUCED GENE SILENCING IN NEUROSPORA CRASSA" NATO ADVANCED SCIENCE INSTITUTES, SERIE H: CELL BIOLOGY,DE,SPRINGER VERLAG, BERLIN, vol. 104, 1998, pages 103-112, XP000906708 ISSN: 1010-8793 --- -/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2000	07/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Ceder, O

2

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P[redacted]ENT COOPERATION TREA[redacted]

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

BANCHETTI, Marina
Ing. Barzanò & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year)
02 November 2000 (02.11.00)

Applicant's or agent's file reference
PCT24285

IMPORTANT INFORMATION

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/IT00/00048	16 February 2000 (16.02.00)	22 February 1999 (22.02.99)

Applicant
UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA" et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BR,BY,CH,CU,DK,DM,EE,ES,FI,GB,GD,GE,GH,GM, HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI, SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

Th International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Zakaria EL KHODARY

Telephone No. (41-22) 338.83.38

Int. tional Application No
PCT/IT 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SHERMAN J M ET AL: "An uncertain silence" TRENDS IN GENETICS,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 13, no. 8, 1 August 1997 (1997-08-01), pages 308-313, XP004084604 ISSN: 0168-9525 ---	
P,X	COGONI : "Neurospora crassa qde-1 gene, partial" EMBL SEQUENCE DATABASE, 10 May 1999 (1999-05-10), XP002144857 HEIDELBERG DE Ac AJ133528 the whole document	1-6, 18-22
P,X	-& COGONI ET AL.: "RNA-dependent RNA polymerase (fragment" EMBL SEQUENCE DATABASE, 1 November 1999 (1999-11-01), XP002144858 HEIDELBERG DE Ac Q9Y7G6 the whole document	1-6, 18-22
P,X	-& COGONI ET AL.: "Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase" NATURE, vol. 399, 13 May 1999 (1999-05-13), pages 166-169, XP002144912 the whole document -----	1-6, 18-22

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

P.D. 1-11-1999
P. Complet (2)

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OC   Eukaryota; Fungi; Ascomycota; Sordariales; Sordariaceae; Neurospora.
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RA   Cogoni C., Macino G.;
RT   "Gene silencing in neurospora crassa requires a protein homologous to

RT   RNA-dependent RNA polymerase.";
RL   Submitted (MAR-1999) to the EMBL/GenBank/DDBJ databases.
DR   EMBL; AJ133528; CAB42634.1; -.
KW   RNA-directed RNA polymerase.
FT   NON_TER    1402   1402
SQ   SEQUENCE   1402 AA;  157982 MW;  FC45A1A17260837D CRC64;

 Q9y7g6  Length: 1402  August 14, 19100 09:46  Type: P  Check: 4314  ..

      1  MNPITPRKRN SPVEEIINRL NNDYNLGLQC VADTTLTPHR RKELAESDED

     51  FGRHDKIYRA LNFLYWRKDD SLNQAEANFF IEAKAASSNW VPKAHADPDT

    101  LPWSKEPPRA ATAGQQWALQ TVLLEVLNRF MPPPNNTPGR TFGRTLSGPS

    151  GLSRPTSTNT KRKDEPANVT FADPPKRSLT RSATGPPIHG AAIPLKFPDP

    201  VNTGSKRPSL ESENLNQCTK RAKGKLSDNV AAAAAPPVPI ASALDKVPTR

    251  RHANTRDPTA TGHRRADQVD SFDTSQGTSY GSSVFSACRH NQSTTQSSFE

    301  APPSQPREKR PVDATVFEAG HLIESPSKGR TTKSHIDNQP LSSSSQGETS

    351  FSTYYESFPS SGGEGAIPEP SRSNGLARSE ESARSQVQVH APVVAARLRN

    401  IWPKFPKWLH EAPLAVAWEV TRLFMHCKVD LEDESLGLKY DPSWSTARDV

    451  TDIWKTLYRL DAFRGKPFPE KPPNDVFVTA MTGNFESKGS AVVLSAVLDY

    501  NPDNSPTAPL YLVKLKPLMF EQGCRLTRRF GPDRFFEILI PSPTSTSPSV

    551  PPVVSKQPGA VEEVIQWLTM GQHSLVGRQW RAFFAKDAGY RKPLREFQLR

    601  AEDPKPIIKE RVHFFAETGI TFRPDVFKTR SVVPAEEPVE QRTEFKVSQM

    651  LDWLLQLDNN TWQPHLKLFS RIQLGLSKTY AIMTLEPHQI RHHKTDLLSP

    701  SGTGEVMNDG VGRMSRSVAK RIRDVLGLGD VPSAVQGRFG SAKGMWVIDV

    751  DDTGDEDWIE TYPSQRKWEC DFVDKHQRTL EVRSVASELK SAGLNLQLLP
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